

Seasonal Amounts of Nutrients in Western Cherry Fruit Fly (Diptera: Tephritidae) and Their Relation to Nutrient Availability on Cherry Plant Surfaces

WEE L. YEE¹ AND PETER S. CHAPMAN

USDA-ARS, Yakima Agricultural Research Laboratory, 5230 Konnowac Pass Rd., Wapato, WA 98951

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ABSTRACT Relatively little is known about the nutritional ecology of fruit flies in the genus *Rhagoletis*. In this study, nutrient amounts in male and female western cherry fruit fly, *Rhagoletis indifferens* Curran, and availability of nitrogen and sugar on surfaces of leaves, fruit, and extrafloral nectaries (EFNs) of sweet cherry trees, were determined from late May to late June 2005 and of sugar from EFNs from mid-May to late June 2007 in Washington state. Protein amounts in male and female flies did not differ over the season. Nitrogen was present on leaves, fruit, and EFNs during the sampling period, but amounts on leaves and fruit were lower in late May than the rest of the season. Sugar amounts in flies did not differ over the season. Sugar was present on leaf, fruit, and EFN surfaces all season, but amounts on all three were lower in late May than later in the season. Fructose and glucose were the predominant sugars on all plant surfaces, but sucrose was also present in nectar from EFNs. In outdoor and field cage experiments in 2004 and 2006, more flies survived when cherry branches with leaves and fruit were present than absent. Results suggest that *R. indifferens* maintains stable protein and sugar levels throughout the season because sufficient amounts of nutrients are found in cherry trees during this time and that increases in nutrient availability caused by ripening and damaged cherries later in the season do not result in increased amounts of nutrients in flies.

KEY WORDS *Rhagoletis indifferens*, nitrogen, sugar, cherry leaves, cherry fruit

Relatively little is known about the nutritional ecology of tephritid fruit flies in the genus *Rhagoletis*, which includes some of the most economically important pests of fruit crops in North America. Information on the nutrient contents of subtropical fruit flies such as the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), has provided insights into the role of nutrients in key fly behaviors. For example, sugar levels were highest in flies during the evening, when most feeding occurs (Warburg and Yuval 1997), and lekking males contained more sugars and protein than resting males, suggesting that reproductive success is linked to foraging success (Yuval et al. 1998). However, the lack of such fundamental information concerning *Rhagoletis* flies has impeded our ability to fully understand the feeding ecology and behaviors of members of this genus. Such questions as where flies find their food, how abundant are foods and nutrients on trees, and whether feeding patterns change as nutrient levels in trees change remain unanswered. Most of what we know about fly nutrition in nature is indirect and derived from observations of feeding behaviors. *Rhagoletis* flies engage in extensive “grazing behaviors” (Hendrichs and Prokopy 1990, Hendrichs et al. 1993, Yee 2008) in which flies rapidly touch their

mouthparts onto a plant surface devoid of any visible food substances but which nonetheless presumably result in feeding. Leachates, which are materials removed from plants through the action of rain, dew, mist, and fog and that include sugars, amino acids, and minerals (Tukey 1970, Mercier and Lindow 2000), are likely ingested by grazing flies. Determining fly nutrient contents can help us better understand fly nutritional ecology, and further explain why flies have evolved particular foraging strategies and behaviors. It also can help interpret results of studies using insecticide-laced food baits for fly control.

The use of nutrients by tephritids for major life activities and the types of substances that flies will feed on are fairly well documented (Drew and Yuval 2000). Proteins or amino acids are essential for maximal egg development (Christenson and Foote 1960) and, in *C. capitata*, for reproductive success in males (Yuval and Hendrichs 2000). Sugar and glycogen serve as fuel for flight, foraging, and courtship in *C. capitata*, and serve as substrates for lipogenesis, and lipids are metabolized to fuel flight, foraging, and courtship (Yuval and Hendrichs 2000). Tephritid flies obtain nutrients from (or at least feed on) fruit juices, extrafloral glandular exudates, flower nectar, pollen, honeydew, bird feces, yeasts, and bacteria (Drew and Yuval 2000, Prokopy and Papaj 2000).

¹ Corresponding author, e-mail: wee.yee@ars.usda.gov.

The western cherry fruit fly, *Rhagoletis indifferens* Curran, is the major insect pest of introduced sweet cherry, *Prunus avium* (L.) L., in the Pacific Northwest of the United States, where its native host is bitter cherry, *Prunus emarginata* Dougl. ex Eaton (Curran 1932). The fly feeds mostly by grazing on nonvisible substances on the surfaces of leaves, but it also feeds on bird feces, cherry juice on leaves and fruit, and on nectar from extrafloral nectaries (Yee 2003, 2008). In central Washington, there is only one major generation of flies each year and most adults are active only for a period of ≈ 4 –5 wk from mid-May to late June (Frick et al. 1954). In sweet cherry trees, the number of leaves with bird feces and cherry juice from fruit injured by birds increases as the season progresses, suggesting that sources of fly nutrients on cherry trees increase over time. However, observations suggest flies feed on nonvisible substances on leaf and fruit surfaces throughout the season (Yee 2008) and that bird feces and cherry juice are not vital for fly nutrition. If true, we would expect *R. indifferens* nutrient reserves to change little during the season.

The objective of this study was to determine the relationship between the nutrient content in *R. indifferens* and the amount of nutrients available to flies on the surfaces of leaves, fruit, and extrafloral nectaries of sweet cherry trees during the season. We tested the hypothesis that nutritional contents of flies remain similar during the season even though nutrient amounts in the environment change over time. We also determined the sugar compositions on cherry leaf, fruit, and extrafloral nectary surfaces, and of nectar from extrafloral nectaries, all of which are nutrient sources for flies. Finally, we determined the ability of flies to survive on cherry tree branches with leaves and fruit alone to support the hypothesis that flies feed on nutrients on plant surfaces.

Materials and Methods

Field Site 2005. Collections of flies, leaves, and fruit for analyses were made in May and June 2005 from three sweet cherry trees (cultivar Bing) in Zillah, WA (46.40° N, 120.26° W). The three trees were growing in residential yards and were situated ≈ 0.4 –4 km from each other. Trees were 5–6.7 m tall, 6–8.3 m wide, and ≈ 20 –30 yr old, were similar in appearance with respect to leaf number and size, and bore high fruit loads. One tree was 3–5 m from mixed fruit trees; another was in a yard with garden plants but no other fruit trees; the third was 10 m from three other fruit trees. Seasonal precipitation and temperature data were obtained from a weather station 3.6–7.8 km away from the trees (AgWeatherNet; [http://weather.wsu.edu]).

Nutrient Amounts in Flies. Flies were captured on leaves and fruit using 5.0 by 1.4-cm glass vials from the three trees once a week. A vial was placed over a fly on a leaf or fruit. Flies on each tree were captured over 1–1.5 h on 23 May and 1, 8, 15, and 22 June between 0900–1400 hours (PST). Vial openings were plugged with cork stoppers immediately after fly capture. Vials

with flies were immediately placed into aluminum or plastic cans surrounded by crushed ice/rock salt in a sealed Styrofoam box (-6 to -10°C) to freeze them in the field. Mean numbers of male flies per tree collected on 23 May and 1, 8, 15, and 22 June were 15.7, 59.7, 51.0, 40.7, and 24.7, respectively (575 total). Mean numbers of female flies collected on these respective dates were 12.3, 39.3, 25.3, 20.3, and 8.7 (318 total). Flies were kept frozen in the field for 2–3 h and were placed in a freezer in the laboratory at -80°C until biochemical analyses to prevent nutrients from degrading. Before analyses, the wing length (from alular notch to wing tip) of each fly was measured using a microscope with an ocular micrometer and the fly's wet weight was recorded on a microbalance (Sartorius, Goettingen, Germany). Protein content of flies was analyzed using Bradford reagent (98%, Acros Organics, Geel, Belgium), sugar and glycogen content using anthrone reagent (Sigma-Aldrich, St. Louis, MO), and lipid content using vanillin (Sigma-Aldrich) in phosphoric acid, as described in Warburg and Yuval (1997) and Yuval et al. (1998), and references therein. Briefly, a fly was crushed in a test tube containing Na_2SO_4 using a glass rod. A chloroform:methanol mixture was added to extract the sugars and lipids. The test tube was centrifuged to separate the sugars and lipids from glycogen and protein, which precipitated out in a pellet. A sample of the supernatant was removed for sugar and another for lipid analysis. For sugar, water and anthrone reagent were added to the sample and heated. Solutions were placed into microplate wells, and optical densities read at 630 nm using a Thermo Multiskan Spectrum spectrophotometer (Thermo Scientific, Milford, MA). For lipids, the sample was dried, dissolved in H_2SO_4 , heated, and mixed with vanillin reagent. Optical densities were read at 530 nm. The pellet containing glycogen and protein was rinsed with methanol. Water was added to the pellet and mixed using a vortex. The mix was allowed to set for 10 min, after which the solids separated from water. For glycogen, part of the water portion was removed and heated, and anthrone reagent added. Optical densities were read at 630 nm. For protein analysis, another part of the water portion was mixed with phosphate-buffered saline (PBS), and PBS was added to the solid portion. Both samples were mixed with Bradford reagent. Optical densities were read at 595 nm. All optical densities were compared with appropriate nutrient standard curves.

Amounts of Nutrients and Sugar Compositions on Plant Parts. During the same times and from the same trees as fly collections, 194–203 leaves, 70–90 fruit (undamaged by birds), and 187–218 additional leaves with extrafloral nectaries (EFNs) were randomly chosen and removed. Leaves were immediately dipped and swished in 200 ml of distilled water in a plastic container for ≈ 5 s. Water drops adhering to leaves were blown into the container using a 12-V battery-operated blower. Fruit and the EFNs on the additional leaves were dipped in beakers containing 30 ml of distilled water. Water has been used to remove sugar from leaf surfaces (Mercier and Lindow 2000), as

sugar is highly soluble in water (Bates 1942). Certain amino acids, which as a group are found on plant surfaces (Tukey 1970), are also water soluble (Dalton and Schmidt 1933). Thus, whereas it is possible that substances adhere to different plant parts differently, at least sugars and amino acids were expected to be removed from leaves and fruit after being dipped in water. The nutrient/water samples were sealed in plastic bottles and frozen at -20 to -80°C for later analyses (below).

Surface areas of leaves, fruit, and EFNs that had been dipped in water were measured or estimated. The surface area of each leaf was measured using a portable area meter (model LI-3000; Lambda Instruments, Lincoln, NE). The weight, percent sugar (measured using a Brix refractometer; ATAGO N1, Tokyo, Japan), and surface area of each fruit was recorded. Each fruit was skinned, its skin spread on a clear acetate sheet, and its area measured with the area meter. The number of exit or respiration holes made by fly larvae on each fruit was recorded. The number of EFNs on each leaf petiole was counted and the dimensions (length and width) of EFNs from 10 randomly selected petioles (per tree/date) were measured to estimate surface areas (EFNs were somewhat rectangular). Seventy-eight to 86% of petioles had two EFNs, 13–21% had one, and 1–2% had three. Thus, one petiole had one EFN or a group of EFNs.

In 2007, nectar from EFNs from three sweet cherry trees in Zillah was collected using 5- μl microcapillary pipettes (VWR International, Leicestershire, United Kingdom) once a week for 7 wk. The objective was to determine nectar volume and sugar compositions over the season. A previous paper (Yee 2008) provided methods and partial data from these nectar collections. On 31 May 2007, honeydew from a colony of black cherry aphid, *Myzus persicae* (Fabricius), was collected from one of the three trees using a microcapillary tube to determine its sugar composition (below).

For nitrogen analyses, portions of leaf, fruit, and EFN dip samples were subjected to the total Kjeldahl nitrogen (TKN) method (AOAC 1985) using a Digesdahl Digestion apparatus (model 23130–20; Hach, Loveland, CO) (Hach Company 1999). This method subjected the samples to a temperature of 440°C in the presence of 96% sulfuric acid (H_2SO_4) and 50% hydrogen peroxide (H_2O_2), which oxidized the carbon to CO_2 and hydrogen to water and which converted amine nitrogen to ammonium ions. These ions were converted into ammonia, which entered a sulfuric acid solution and was converted back into ammonium ions. The ammonium ions in solution were reacted with Nessler's reagent (potassium tetraiodomercurate in potassium hydroxide), which resulted in light yellow to yellow solutions, depending on the amount of ammonium ions. Light intensities of the reacted samples were measured with a Thermo Multiskan Spectrum spectrophotometer set at a wavelength of 425 nm and compared with ammonia nitrogen (100 mg/liter) standards (Hach). The conversion of nitrogen to protein requires a specific factor characteristic of plants or

animals (Levey et al. 2000). In our study, most of the nitrogen was probably from cherry trees, but some also may have been from nonprotein nitrogen such as uric acid. Thus, results are presented simply as "nitrogen" instead of protein.

For sugar analyses, the remaining portions of leaf, fruit, and EFN dip samples were filtered using a syringe filter (Target, Nylon 0.2- μm pore size; National Scientific Co., Rockwood, TN) to remove particulate matter. A volume of 5, 10, or 20 μl of samples was injected into an Agilent 1100 Series high-performance liquid chromatography (HPLC) with a refractive index detector and a Zorbax Carbohydrate column (4.6 by 250 mm; 5 μm ; Agilent Technologies, Palo Alto, CA). A 75% acetonitrile, 25% HPLC water ("Baker Analyzed" HPLC Reagent; Mallinckrodt Baker, Phillipsburg, NJ) solvent was used in the HPLC to separate out sugars. Amounts and identities of the sugars were compared using glucose, fructose (Sigma-Aldrich), and sucrose standards (Acros Organics, Geel, Belgium). Glucose, fructose, and sucrose are known to be present in cherry fruit (Lee et al. 1970). For analyses of nectar and the honeydew sample from 2007, similar methods were used, except that 100 μl of HPLC water was used to extract the nectar or honeydew from the microcapillary tubes used for collections and only 2 μl was injected into the HPLC.

Survival of Flies Exposed to Leaves and Fruit 2004 and 2006. To show that flies likely obtained nutrients from surfaces of leaves, fruit, and/or EFNs, an outdoor cage experiment was conducted in 2004 at the USDA Yakima Agricultural Research Laboratory in Wapato, WA (46.28°N , 120.22°W). Cherry branches with leaves and fruit were removed from trees (cultivar Bing) at the USDA experimental orchard near Moxee, WA (46.29°N , 120.10°W) and exposed to flies. Three tests were conducted, each for 3 d during (1) 27–30 May, (2) 4–6 June, and (3) 15–18 June 2004. In each test, 34–40 field-collected flies (8 females, 26–32 males) that had been held on 20% sucrose (wt:wt) for 3 d were released inside a plywood cage (33 cm wide by 53.5 cm long) with organically screened held outdoors in the shade next to the laboratory. For (1), there was a control (no branch), one branch, and five branch treatment; for (2), there were 0, 1, 2, and 5 cherry branch treatments; to provide resting sites and additional shade, there were 5, 4, 3, and 0 artificial branches with silk leaves (Silk Gardens Shop, Irving, TX), respectively. For (3), treatments were the same as (2), except the five cherry branch treatment was omitted. There was one replicate per treatment for each test. In test (1), numbers of leaves:fruit were 104:46, 213:182, and 510:259 for 1, 2, and 5 branch treatments, respectively; in test (2), 124:1, 205:2, and 528:15 for 1, 2, and 5 branch treatments, respectively. Leaf and fruit numbers were not recorded for test (3), but branches had few fruit. Deionized water was provided on cotton wicks. All fruit were undamaged and there were no aphids on any treatment except for small numbers on the one branch treatment in (2). Branches were inserted into 1-liter flasks filled with deionized water. The number of dead and live flies was

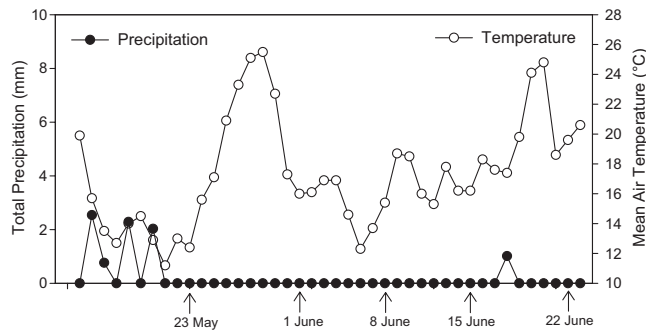


Fig. 1. Seasonal precipitation and temperatures at a weather station 3.6–7.8 km from study trees in Zillah, WA, 2005. Dates shown with arrows were sample dates.

counted after 3 d. Temperature and humidity over 3 d ranged from 5 to 34, 9 to 30, and 9 to 27°C and 34–100, 40–100, and 30–100% RH for the three respective tests.

To provide further evidence that flies obtained nutrients from leaves, fruit, and/or EFNs, a field experiment using cages placed over branches on trees was conducted in 2006 at the USDA experimental orchard

near Moxee. Tests were conducted during 23–30 May, 6–13 June, and 22–29 June 2006. Flies used in the experiment were collected as larvae from cherry fruit in central Washington in June to July 2005 and chilled at 3 and 11°C for ≈9 mo until May 2006, when they were transferred to 27°C for adult emergence. Flies were kept on 5% sucrose (wt:wt) and deionized water

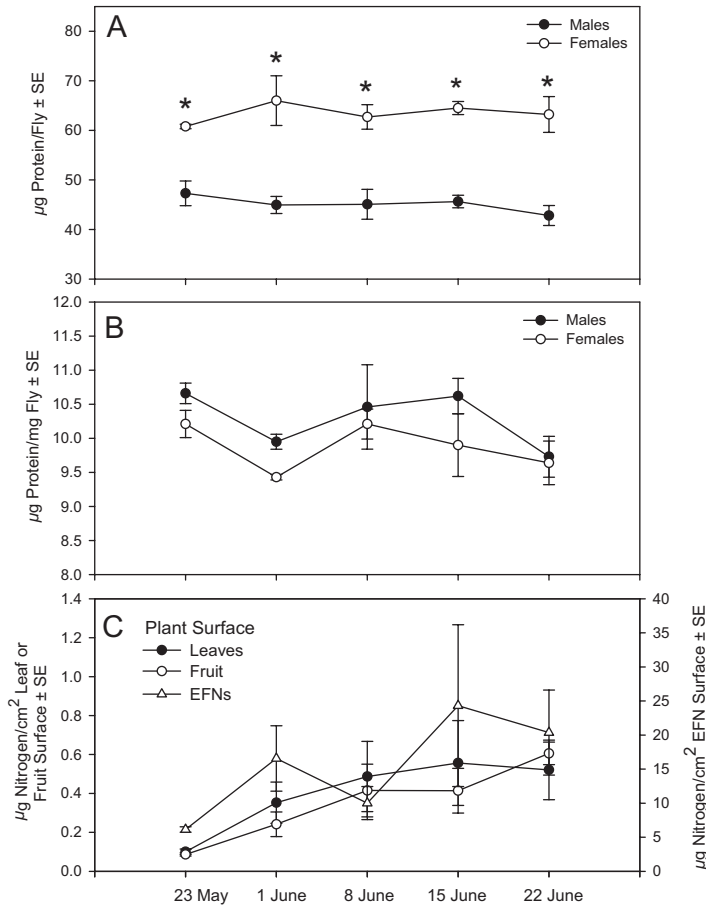


Fig. 2. Mean amounts of protein in (A) male and female *R. indifferens*, (B) in males and females per mg body weight, and (C) amounts of nitrogen on surfaces of cherry plant parts in Zillah, WA, 2005. *Significant sex differences within dates (one-way ANOVA, $P < 0.05$).

Table 1. Repeated-measures ANOVA of date effects on amounts of protein in *R. indifferens* and nitrogen on plant surfaces, Zillah, WA, 2005

		Males		Females	
	<i>F</i> ^a	<i>P</i>		<i>F</i> ^a	<i>P</i>
Protein/fly	0.9		0.4993	0.6	0.6757
Protein/mg fly	1.6		0.2560	1.2	0.3994
Amounts of nitrogen on plant surface					
Plant Part	<i>F</i> ^a	<i>P</i>	Significant pairwise comparisons	<i>F</i> ^b	<i>P</i>
Leaves	6.3	0.0137	23 May versus 1 June	19.7	0.0022
			Versus 8 June	17.7	0.0030
			Versus 15 June	17.1	0.0033
			Versus 22 June	14.1	0.0056
Fruit	12.3	0.0017	23 May versus 1 June	22.5	0.0015
			Versus 8 June	32.4	0.0005
			Versus 15 June	27.5	0.0008
			Versus 22 June	41.4	0.0002
			1 June versus 8 June	6.5	0.0342
			1 June versus 22 June	12.4	0.0079
			15 June versus 22 June	5.1	0.0531
EFNs	2.4	0.1329	—	—	—

^a Repeated-measures ANOVA, df = 4,8.

^b Orthogonal contrasts, df = 1,8.

until 24 h before release into cages. Five instead of 20% sucrose was used to increase the chance that survival was affected more by exposure to nutrients on leaves, fruit, and/or EFNs than by energy reserves accumulated from heavy sugar feeding before the experiment. Flies were 3–6 d old. There were three treatments, with five male and five female flies per cage: (1) water (deionized) only, (2) branches with fruit and leaves, and (3) sucrose only. Leaves and fruit surfaces were examined to ensure cherry juice and aphids were absent. Treatments were randomly assigned to trees. Cages (30 by 30 by 30 cm) were placed in the crotches beneath the canopies of 3–4 m tall sweet cherry trees (cultivar Bing) to provide ample shade. Each cage had an acrylic plastic bottom, four sides with window screen (1-mm² openings), and one side with a cloth sleeve. The cage was tightly secured to branches below or above it using duct tape. For treatment (1), no branch was inserted into the cage, and only water was provided. For treatment (2), a branch was inserted into a cage, whose cloth sleeve was then tied to the branch. For treatment (3), ≈50 ml of 5% sucrose (wt:wt) was squirted onto a sheet of cotton on a plastic dish (14.8 cm diameter by 1.3 cm high) on the cage bottom. There was no branch in the cage. In each cage, deionized water from a 550-ml container was soaked onto a sheet of cotton. Each of the three treatments was set up in five replicate trees. Different trees were used in the three periods. At the end of 7 d, cages and branches were removed from trees and live and dead flies counted.

Fruit in treatment (2) during first, second, and third periods were green, yellow, and red, respectively. In the first period, number of fruit, fruit weight, percent sugar, number of leaves, and total leaf area (mean ± SE) were 51.8 ± 16.4, 1.37 ± 0.03 g, not detected, 87.4 ± 11.6, and 3,167.2 ± 375.6 cm², respectively. In the second period, these were

23.4 ± 8.2, 2.79 ± 0.28 g, 8.4 ± 0.3%, 64.0 ± 14.8, and 2,525.1 ± 655.8 cm². In the third period, these were 33.6 ± 8.0, 6.45 ± 0.20 g, 15.9 ± 0.3%, 58.6 ± 3.2, and 2,233.5 ± 218.7 cm². Mean low and high temperatures during the three respective periods were 20.1 and 5.6, 23.6 and 9.1, and 32.1 and 10.6°C.

Statistical Analyses. Repeated-measures analysis of variance (ANOVA) was used to analyze body size and seasonal nutrient amounts within male and female flies, using each tree as a replicate. Sex could not be included as a factor in this ANOVA because male and female flies were from the same three subjects (trees). Repeated-measures ANOVA was also used to determine whether nitrogen and sugar amounts within leaves, fruit, and EFNs differed over the season, and whether percent sugar in fruit and fruit weight differed over the season. To reduce the variance, nitrogen and sugar values were subjected to logarithmic and logarithmic ($y + 0.5$) transformation (because of zeroes), respectively. Orthogonal contrasts (Yandell 1997) were used to test for differences among all 10 pairwise comparisons of the five dates. One-way ANOVA was performed within each date to determine differences in nutrients between male and female flies and in nitrogen or sugar amounts among different plant parts. For the 2004 cage test, there was one replicate, so a Tukey-type multiple comparison test among proportions (Zar 1999) was used to determine differences in percent flies surviving among treatments. For the 2006 field cage test, two-way ANOVA was used to analyze percent flies surviving (treatment and date as factors). One- and two-way ANOVAs were followed by a Fisher least significant difference (LSD) test. Percentages were square-root and arcsine transformed before analyses. Data were analyzed using PROC MIXED and PROC GLM in SAS (SAS Institute 2004).

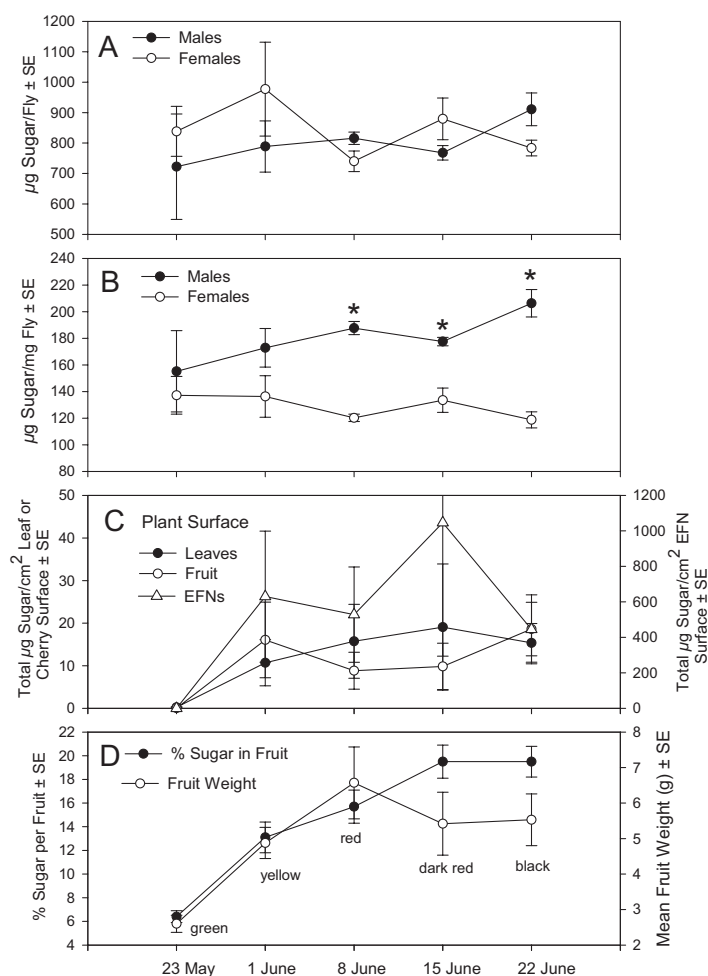


Fig. 3. Mean amounts of sugar in (A) male and female *R. indifferens*, (B) in males and females per mg body weight, (C) on surfaces of cherry plant parts, and (D) percent sugar in fruit, fruit weight, and color of majority of fruit in Zillah, WA, 2005. *Significant sex differences within dates (one-way ANOVA, $P < 0.05$).

Results

Seasonal Precipitation and Temperatures. Precipitation was overall low, with only 0.76–2.54 mm on each of 4 d for a total of 7.62 mm during the week before the first sample date of 23 May 2005. There was no precipitation 2 d before 23 May and none afterward except for 1.02 mm on 18 June (Fig. 1). The mean air temperatures were low most of the week before 23 May, but they increased afterward, although there was much fluctuation (Fig. 1).

Protein Amounts in Flies and Amounts of Nitrogen on Plant Parts. There was no seasonal effect on the wing lengths of male flies ($F = 0.3$; $df = 4,8$; $P = 0.8928$), although there was a seasonal effect on the wing length of female flies ($F = 4.8$; $df = 4,8$; $P = 0.0294$). Wing lengths of females on 1, 8, and 15 June were longer than those on 22 June 2005 (orthogonal contrasts, $F = 8.7$ – 13.7 ; $df = 1,8$; $P = 0.0186$ – 0.0060). However, there was no significant seasonal effect on body weights of males ($F = 2.0$; $df = 4,8$; $P = 0.1925$)

and females ($F = 3.1$; $df = 4,8$; $P = 0.0841$). Wings of males and females were 2.970 ± 0.003 and 3.312 ± 0.016 mm long ($F = 816.1$; $df = 1,4$; $P < 0.0001$), respectively, and body weights were 4.412 ± 0.042 and 6.539 ± 0.177 mg ($F = 95.0$; $df = 1,4$; $P = 0.0006$), respectively.

Hemolymph dissolved protein was measured using methods described in this study (Yuval et al. 1998). There were no seasonal effects on protein amounts in male or female flies (Fig. 2A and B; Table 1). Amount of protein in female flies was greater than in male flies (Fig. 2A), but males contained almost significantly more protein than females per body weight (Fig. 2B). In contrast to protein amounts in flies, the µg nitrogen/cm² on leaves, fruit, and EFNs increased as the season progressed, although the increase on EFNs was not significant (Fig. 2C; Table 1). There was more nitrogen/cm² on EFNs than leaves and fruit on each of the five dates (one-way ANOVA, $F = 25.3$ – 571.7 ; $df = 2,6$; $P = 0.0012$ to $P < 0.0001$).

Table 2. Repeated-measures ANOVA of date effects on amounts of sugar in *R. indifferens* and sugar on plant surfaces, Zillah, WA, 2005

		Males		Females	
		<i>F</i> ^a	<i>P</i>	<i>F</i> ^a	<i>P</i>
Sugar/fly		1.0	0.4699	1.6	0.2594
Sugar/mg fly		1.8	0.2212	1.0	0.4511
Amounts of sugar on plant surface					
Plant Part	<i>F</i> ^a	<i>P</i>	Significant pairwise comparisons	<i>F</i> ^b	<i>P</i>
Leaves	3.9	0.0494	23 May versus 1 June	10.0	0.0134
			Versus 8 June	10.6	0.0117
			Versus 15 June	9.3	0.0160
			Versus 22 June	10.9	0.0108
Fruit	4.6	0.0327	23 May versus 1 June	12.5	0.0077
			Versus 8 June	9.2	0.0164
			Versus 15 June	8.2	0.0212
			Versus 22 June	13.1	0.0068
EFNs	4.2	0.0392	23 May versus 1 June	11.7	0.0090
			Versus 8 June	7.3	0.0269
			Versus 15 June	12.2	0.0081
			Versus 22 June	9.4	0.0154

^a Repeated-measures ANOVA, df = 4,8.
^b Orthogonal contrasts, df = 1,8.

Sugar Amounts in Flies and Amounts of Sugar on Plant Parts. There were no seasonal effects on sugar amounts in male or female flies (Fig. 3A and B; Table 2). The amounts of sugar in female and male flies did not differ (Fig. 3A). However, per body weight, there was more sugar in males on the last three dates (Fig. 3B). In contrast to the lack of seasonal differences in sugar amounts in flies, the μg sugar/ cm^2 on leaves, fruit, and EFNs were higher on 23 May than on all other dates (Fig. 3C; Table 2). There were no differences in amounts of sugar/ cm^2 on surfaces of leaves, cherries, and EFNs on 23 May, 1 June, and 8 June 2005 ($P > 0.05$), but the amounts were higher on EFNs than on leaves and fruit on 15 and 22 June (one-way ANOVA, $F = 9.3$ and 9.5 ; $\text{df} = 2,6$, $P = 0.0145$ and 0.0138 , respectively). The percent sugar in fruit increased as the season progressed ($F = 30.1$; $\text{df} = 4,8$; $P < 0.0001$), with differences between all dates except 15 and 22 June ($P > 0.05$; Fig. 3D). Fruit weight increased until 8 June, after which it leveled off ($F = 17.1$; $\text{df} = 4,8$; $P = 0.0005$; Fig. 3D). In 2007, nectar was consistently available over the season (Fig. 4), al-

though there were no increases over time in percent of EFNs or groups of EFNs with detectable nectar, nor were there increases in nectar volume ($P > 0.05$).

Sugar Compositions on Plant Parts. Sugars on leaf surfaces on 23 May were likely those leached from leaves and not from cherry juice because fruit on this date was hard and green, and juice was not seen on leaves. On leaf surfaces on this date, the predominant sugar was fructose, although glucose also occurred as a large percentage (Table 3). No sucrose was detected. On three of the four remaining dates, glucose was the predominant sugar (Table 3). On fruit surfaces, glucose and fructose were detected, but sucrose was not. Except on 23 May, the percentage of glucose was higher than that of fructose (Table 3). HPLC chromatograms showed no large peaks other than for glucose and fructose. There were no holes in fruit caused by larvae on 23 May and 1 and 8 June, but on 15 and 22 June 2005, there were 0.5 ± 0.1 and 0.6 ± 0.2 holes, respectively, so some sugars on the last two dates may have been derived from inside fruit.

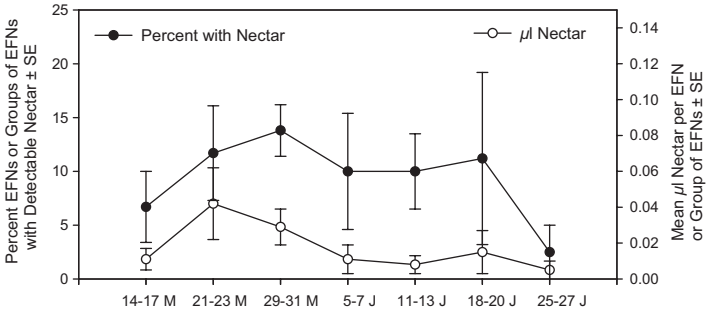


Fig. 4. Mean seasonal abundance of nectar from EFNs of cherry trees in Zillah, WA, 2007. An EFN or group of EFNs consisted of one to three EFNs on one leaf petiole. M, May; J, June.

Table 3. Seasonal amounts and percent compositions of sugars (means \pm SE) on leaf and fruit surfaces of cherry trees in which *R. indifferens* were collected in Zillah, WA, 2005

Date	Surface area/leaf ^a or fruit (cm ²)	Glucose	Fructose	Sucrose
Leaf surfaces: μg sugars/cm ² (% composition)				
23 May (0/3) ^b	81.9 \pm 3.2	0.10 \pm 0.01 (40.6)	0.14 \pm 0.07 (59.4)	ND
1 June (2/3) ^b	85.5 \pm 4.9	6.4 \pm 3.3 (45.6)	4.3 \pm 2.1 (54.4)	ND
8 June (3/3) ^b	83.9 \pm 5.2	9.4 \pm 5.2 (58.4)	6.4 \pm 3.5 (41.6)	ND
15 June (3/3) ^b	79.3 \pm 7.6	11.6 \pm 9.1 (60.1)	7.4 \pm 5.7 (39.9)	ND
22 June (3/3) ^b	71.6 \pm 2.7	9.2 \pm 2.6 (59.4)	6.2 \pm 1.9 (40.6)	ND
Fruit surfaces: μg sugars/cm ² (% composition) ^c				
23 May	7.5 \pm 0.5	0.01 \pm 0.01 (24.1)	0.02 \pm 0.02 (75.9)	ND
1 June	10.9 \pm 1.0	9.1 \pm 4.9 (56.3)	7.2 \pm 4.1 (43.7)	ND
8 June	11.4 \pm 1.3	4.8 \pm 2.3 (55.5)	4.0 \pm 2.0 (44.5)	ND
15 June	12.8 \pm 1.5	5.3 \pm 3.0 (52.5)	4.5 \pm 2.4 (47.5)	ND
22 June	14.1 \pm 1.5	9.9 \pm 3.5 (51.9)	8.7 \pm 2.8 (48.1)	ND

^a Based on 194–203 leaves from each of three trees; includes tops and bottoms of leaves.

^b Numbers of trees out of three that had cherry juice stains on their leaves.

^c Based on 70–90 fruit from each of three trees.

ND, not detected.

Extrafloral nectaries in 2005 had sugars on their surfaces, but drops of nectar were not visible. The predominant sugar in EFN dip samples was glucose, followed by fructose, but sucrose was not detected (Table 4). In contrast, in 2007, the predominant sugar in nectar collected from EFNs was sucrose (especially high in late season), which were followed by glucose and fructose, both similar in percentages (Table 4).

The surface area of a set of EFNs on a petiole in 2005 was \approx 3,000 times smaller than the area of a leaf and \approx 250–530 times smaller than that of a fruit, whereas a fruit's surface area was \approx 5–11 times smaller than that of a leaf (Tables 3 and 4). Thus, EFNs occupied a small area, but had high concentrations of fructose and glucose on their surfaces.

Black cherry aphid honeydew on 31 May 2007 contained 19.1% glucose, 58.3% fructose, and 22.6% sucrose. However, there were three unidentified peaks

in the HPLC chromatogram. These probably represented other sugars, and one was more than twice as abundant as fructose.

Glycogen and Lipid Amounts in Flies. There were no seasonal effects on total glycogen in male or female flies (Fig. 5A; Table 5). However, there were seasonal effects on glycogen/mg fly in males, with amounts lowest on 23 May, although not in females (Fig. 5B; Table 5). Amounts of glycogen in females were greater than in males (Fig. 5A), but per body weight there were no differences (Fig. 5B). There were seasonal effects on total lipid and lipid/mg fly in male and female flies, because lipid amounts were lowest on 22 June (Fig. 5C and D; Table 5). Lipid amounts in females were greater than in males, but per body weight, there were no differences between females and males, and lipids in both sexes declined at the end of the season (Fig. 5C and D).

Table 4. Seasonal amounts and percent compositions of sugars (means \pm SE) from EFNs of cherry leaves in Zillah, WA, 2005 and 2007

EFN surfaces (dipped in water): μg sugar/cm ² (% composition) (2005)				
Dates	Surface area/EFN ^a (cm ²)	Glucose	Fructose	Sucrose
23 May	0.0253 \pm 0.0006	0 \pm 0	0 \pm 0	ND
1 June	0.0285 \pm 0.0039	383.8 \pm 225.7 (60.7)	246.6 \pm 143.5 (39.3)	ND
8 June	0.0280 \pm 0.0003	312.9 \pm 158.2 (59.4)	215.1 \pm 110.2 (40.6)	ND
15 June	0.0237 \pm 0.0039	647.1 \pm 483.5 (55.8)	398.8 \pm 268.8 (44.2)	ND
22 June	0.0285 \pm 0.0032	263.8 \pm 120.4 (55.0)	181.2 \pm 74.3 (45.0)	ND
Nectar collected from EFNs: μg sugar/ μl nectar (% composition) (2007)				
Dates	Glucose	Fructose	Sucrose	
14–17 May	149.3 \pm 7.1 (18.7)	165.4 \pm 6.1 (20.9)	478.9 \pm 51.0 (60.4)	
21–23 May	165.1 \pm 52.3 (19.3)	176.8 \pm 52.3 (21.4)	534.8 \pm 202.2 (59.3)	
29–31 May	144.1 \pm 8.1 (14.8)	157.1 \pm 8.1 (16.1)	670.2 \pm 40.1 (69.1)	
5–7 June	220.9 \pm 47.7 (15.6)	237.6 \pm 52.3 (16.6)	893.4 \pm 343.3 (67.8)	
11–13 June	104.3 \pm 14.9 (13.2)	113.9 \pm 13.8 (14.6)	556.5 \pm 63.8 (72.2)	
18–20 June	148.2 \pm 10.9 (12.1)	155.0 \pm 13.8 (13.0)	951.2 \pm 163.7 (74.9)	
25–27 June	90.6 \pm 9.4 (9.5)	102.7 \pm 9.1 (10.5)	796.2 \pm 82.8 (80.0)	

2005, means from three trees; 2007, means from three or four trees.

^a Per EFN or group of EFNs per petiole; based on 187–218 leaf petioles from each of three trees.

ND, not detected.

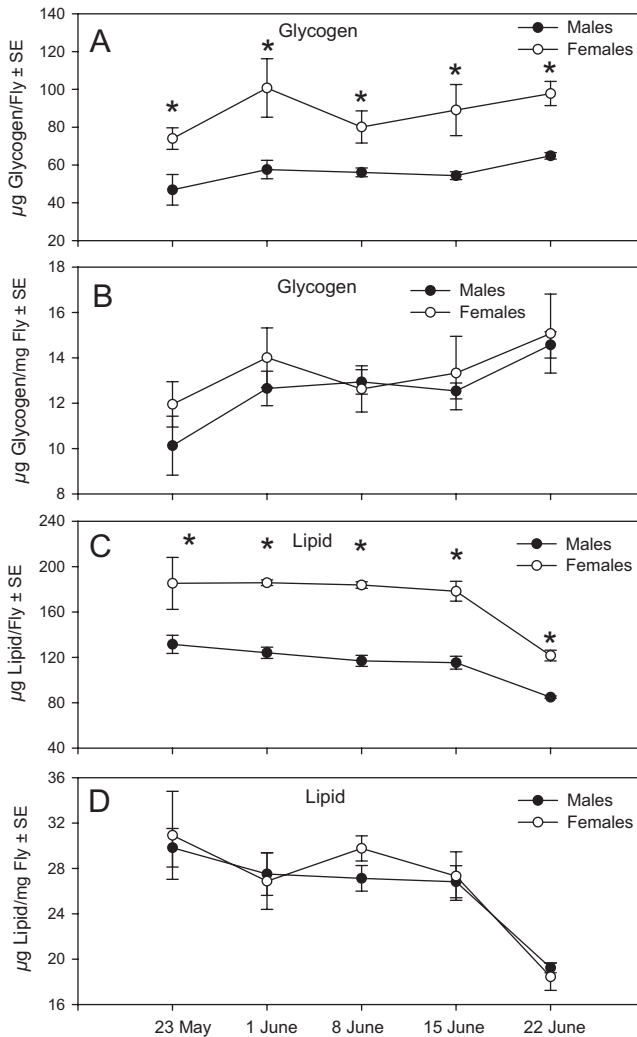


Fig. 5. Mean amounts of glycogen in (A) male and female *R. indifferens* and (B) in males and females per mg body weight. Amounts of lipid in (C) male and female *R. indifferens* and (D) in males and females per mg body weight in Zillah, WA, 2005. *Significant sex differences within dates (one-way ANOVA, $P < 0.05$).

Survival of Flies Exposed to Leaves and Fruit. In the first test in 2004, percent survival of flies exposed to five branches was higher than of those exposed to no branch or one branch ($q = 1.22$ – 4.50 ; critical $q_{0.05, \infty, 3} = 3.314$; Fig. 6A). In the second test, survival of flies exposed to one, two, and five branches was higher than that exposed to no branch ($q = 1.43$ – 9.22 ; critical $q_{0.05, \infty, 4} = 3.633$; Fig. 6B). In the third test, survival of flies exposed to one and two branches was higher than that exposed to no branch ($q = 3.32$ – 7.54 ; critical $q_{0.05, \infty, 3} = 3.314$; Fig. 6C).

In 2006, survival of flies differed during the three periods (Fig. 7A–C). There were treatment ($F = 31.0$; $df = 2, 35$; $P < 0.0001$) and period effects ($F = 7.6$; $df = 2, 35$; $P = 0.0018$) and no treatment \times period interaction ($F = 0.6$; $df = 4, 35$; $P = 0.6635$). Survival was highest in the sucrose treatment, followed by the branch and water treatment ($P < 0.05$). Within the branch treatment

across the three periods, 23 and 35 live males and females, respectively, were recovered ($\chi^2 = 2.48$; $df = 1$; $P = 0.1151$). Within the sucrose treatment across the three periods, 38 live males and 56 live females were recovered ($\chi^2 = 3.45$; $df = 1$; $P = 0.0634$). Leaves, fruit, and EFNs were devoid of visible food substances.

Discussion

There were no seasonal effects on the protein and sugar amounts in male and female *R. indifferens*, whereas after late May, the amounts of nitrogen and sugar on leaf, fruit, and EFN surfaces of cherry trees generally were greater. Whether nutrient contents of flies are affected by differences in metabolism because of seasonal temperature changes and whether precipitation affects nutrient levels on leaves need to be determined. However, our results suggest that flies maintain stable protein and

Table 5. Repeated-measures ANOVA of date effects on amounts of glycogen and lipid in *R. indifferens*, Zillah, WA, 2005

Glycogen/fly	<i>F</i> ^a	<i>P</i>	Significant pairwise comparisons	<i>F</i> ^b	<i>P</i>
Males	3.5	0.0618	—	—	—
Females	1.5	0.2996	—	—	—
Glycogen/mg fly			—	—	—
Males	5.6	0.0189	23 May versus 1 June	10.6	0.0116
			Versus 8 June	8.5	0.0194
			Versus 15 June	5.2	0.0513
			Versus 22 June	16.3	0.0037
			15 June versus 22 June	6.9	0.0302
Females	0.8	0.5730	—	—	—
Lipid/fly					
Males	10.7	0.0027	23 May versus 22 June	37.4	0.0003
			1 June versus 22 June	26.5	0.0009
			8 June versus 22 June	18.2	0.0028
			15 June versus 22 June	19.0	0.0024
Females	6.1	0.0147	23 May versus 22 June	15.7	0.0042
			1 June versus 22 June	15.9	0.0040
			8 June versus 22 June	15.2	0.0046
			15 June versus 22 June	11.1	0.0105
Lipid/mg fly					
Males	8.9	0.0048	23 May versus 22 June	28.8	0.0007
			1 June versus 22 June	18.0	0.0028
			8 June versus 22 June	17.6	0.0030
			15 June versus 22 June	21.2	0.0017
Females	4.3	0.0388	23 May versus 22 June	13.6	0.0062
			1 June versus 22 June	6.2	0.0375
			8 June versus 22 June	11.5	0.0096
			15 June versus 22 June	6.0	0.0397

^a Repeated-measures ANOVA, df = 4,8.^b Orthogonal contrasts, df = 1,8.

sugar levels because of the consistent availability of nitrogen and sugar on cherry trees throughout the season. Flies do visit nonhost trees to feed, but because feeding on cherry trees is so frequently observed (Yee 2008), it is likely most feeding occurs on host trees. In addition, our one study tree that was near garden plants (which flies have not been seen feeding on) had flies with similar nutrient levels as flies from the other two trees. Whether flies preferentially feed on different foods on cherry trees over the season needs to be examined. The possibility that female flies consuming more nutrients could transfer nutrients to dispersed eggs so that nutrients in their bodies do not reflect consumption of nutrients also needs to be examined.

Sugar was 26, 28, and 31 times more abundant than nitrogen on leaves, fruit, and EFNs, respectively (based on $\mu\text{g}/\text{cm}^2$ plant surface) and ≈ 5 times more abundant if nitrogen is converted to plant protein [\times factor of 6.25 (Levey et al. 2000)]. Thus, sugar was probably more easily obtained than nitrogen just through indiscriminate feeding on leaf surfaces. In *R. pomonella*, sugar is ingested daily and frequently (Hendrichs et al. 1990), and this is probably true in all *Rhagoletis*.

There was no seasonal effect on protein amounts in male and female *R. indifferens*. Flies contained 45–65 μg protein throughout the season, although how much of this originated from recent meals is unclear. However, *R. pomonella* consumed 11–180 μg protein/fly/d (Webster et al. 1979) [yeast extract is $\approx 62\%$ protein (Sigma Life Science 2008)], so our results are within expectations of protein ingestion given that *R. indifferens* is smaller than *R. pomonella*. Hemolymph protein contents also suggest

C. capitata feeds on constant amounts of protein throughout life (Nestel et al. 2005).

Although no seasonal effect on sugar amounts in flies were detected, male *R. indifferens* had higher amounts of sugar per body weight than females later in the season. Males may have fed more frequently on sugar than females later in the season or males used sugar less quickly than the more active females. Males spend much time waiting on fruit for mates, whereas females spend much time moving back and forth between fruit and leaves, apparently looking for food and oviposition sites (Yee 2002). The apparently diffuse amounts of sugar early in the season suggest both sexes need to search and graze many leaves to find them. This is suggested by the fact that on 23 May 2005, cherry juice was not a sugar source and that there was only a total of 0.24 μg of sugar/ cm^2 of leaf, yet female flies already had 839 μg of sugar, similar to later in the season.

Seasonal foraging frequency by *R. indifferens* on different plant parts (Yee 2008) may be explained in part by the surface areas of the parts rather than the concentration of nutrients on them. The amounts of nitrogen and sugar/ cm^2 on leaf and fruit surfaces were similar, but more flies grazed leaves than fruit; furthermore, EFNs had the most nitrogen and sugar/ cm^2 , but relatively few flies fed on them (Yee 2008). These surface area/sugar relationships may also be similar in the fly's native host, bitter cherry. Aphid honeydew has not been confirmed as a food in nature possibly because it occupies low surface areas.

There were probably several sources of nitrogen and sugars in our study. Nitrogen on leaves probably

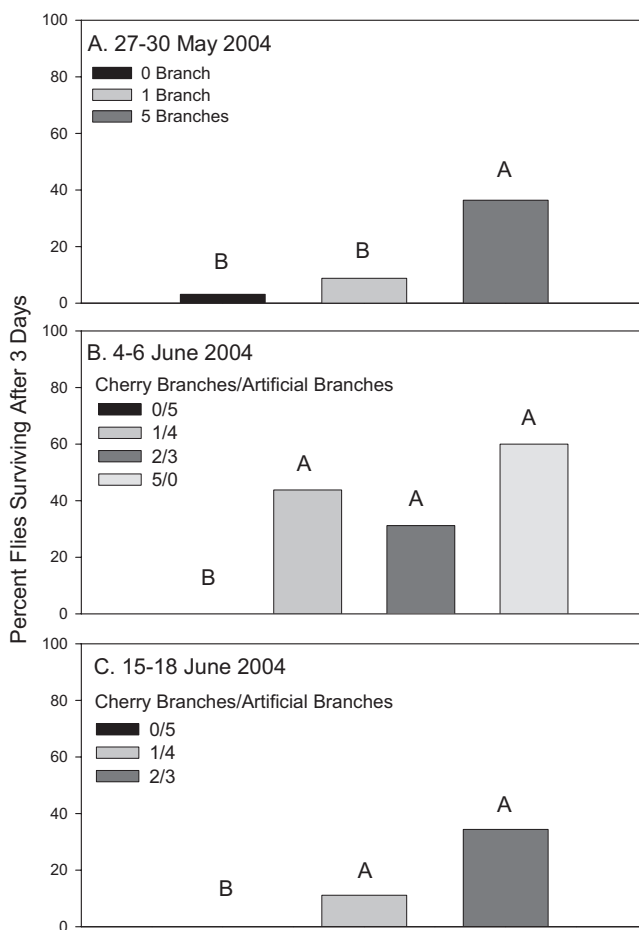


Fig. 6. Survival of *R. indifferens* exposed to different numbers of branches (with leaves and fruit) from cherry trees after 3 d in outdoor cages in Wapato, WA, 2004: (A) test 1, (B) test 2, and (C) test 3 periods. Percentages with same letters are not significantly different (Tukey-type multiple comparison test among proportions, $P > 0.05$).

originated from cherry juice, which is $\approx 1\%$ protein (USDA 2005), bird feces (uric acid), and amino acids in leachates (Tukey 1970). Bacteria and yeasts (Lauzon et al. 2003) also may have contributed some nitrogen. Nitrogen from EFNs probably originated from amino acids. In EFNs of castor bean, low amounts of protein, glutamic acid, and nitrate have been found (Baker et al. 1978). The main source of fructose and glucose on cherry leaf surfaces on our first sample date was probably leachates (Tukey 1970), but on remaining dates the sources were probably cherry juice and leachates. Sugars from EFNs were from nectar, but there apparently are differences in composition between sugars leached to surfaces of EFNs and those actively secreted by them. Dips of EFNs in water in 2005 did not yield sucrose, whereas EFN nectar in 2007 consistently yielded sucrose. In nectar from EFNs of castor bean and cow pea, as in cherry EFN dips, fructose and glucose were abundant, even though the phloem sap of these plants is $>95\%$ sucrose (Baker et al. 1978, Pate et al. 1985). Interestingly, sucrose is a highly stimulating sugar for *R. indifferens*, yet it was

absent in cherry trees throughout the season except in EFN nectar. In *R. pomonella*, sucrose and fructose were more stimulating than glucose, melezitose, and maltose (Duan and Prokopy 1993).

Seasonal amounts of sugar and glycogen in flies followed similar patterns, reflecting the conversion of sugar to glycogen, but data suggest that the sugar was not converted efficiently into lipids by either sex at the end of the season. This result suggests flies with high sugar/glycogen and low lipid amounts were nearing the end of their lifespan. Sugar was abundant at the end of the season, but fly numbers during this time were low. In *C. capitata*, lipid contents significantly dropped at very advanced ages (Nestel et al. 2005).

Results from the 2004 and 2006 cage experiments suggest male and female *R. indifferens* survived on the nutrients on surfaces of cherry leaves, undamaged fruit, and EFNs, which almost certainly included nitrogen, glucose, and fructose, regardless of the time of season. In May, leaf surfaces in particular were likely sources of nutrients for flies because fruit during this month were hard and green or yellow, with low or no

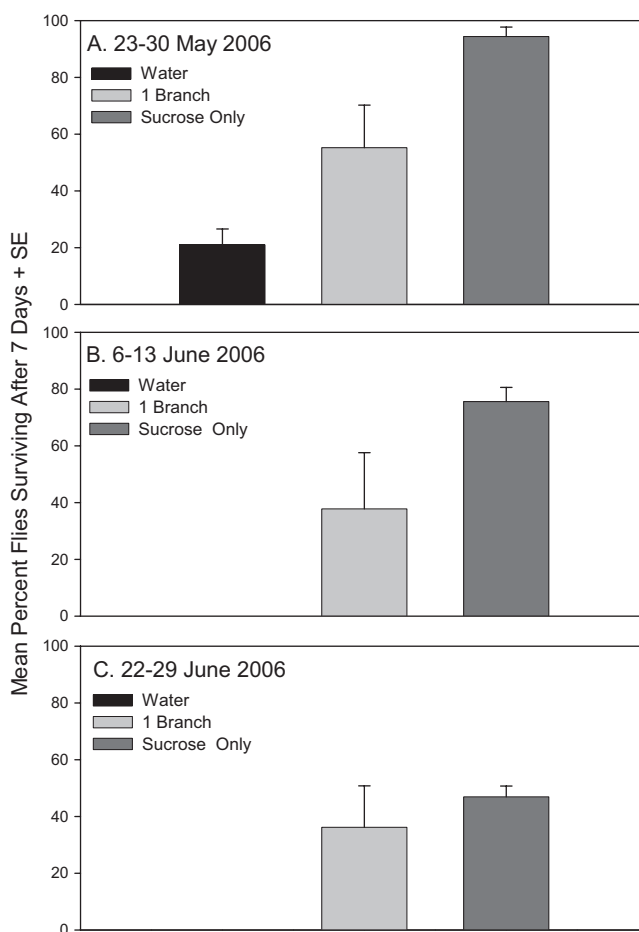


Fig. 7. Mean survival of *R. indifferens* exposed to branches (with leaves and fruit) in cages on cherry trees after 7 d near Moxee, WA, 2006: (A) first, (B) second, and (C) third periods. Overall means among water, one branch, and sucrose only treatments were all significantly different, according to two-way ANOVA (no treatment \times period interaction, LSD test, $P < 0.05$).

detectable sugar. Similar to *R. indifferens* on cherry, *R. pomonella* exposed to hawthorn and apple leaves obtained sufficient nutrients to sustain some survival (Hendrichs et al. 1993).

The results have implications for the management of *R. indifferens*. Control of flies using insecticide baits may be effective because flies forage over many leaves to obtain diffuse food, such that baits need not be attractive to be found and ingested (Yee and Chapman 2005). It is possible that high cherry juice abundance later in the season can result in competition with insecticide baits. Discouraging birds from attacking fruits has been mentioned as a possible way to decrease fruit juice available to flies (Hendrichs and Prokopy 1990). Food abundance may also affect fly dispersal. If cherry trees provide flies with all the nutrients they need, dispersal from unmanaged, infested trees to commercial trees should occur at low levels as long as fruit are present and fly infestations are not too high. In *R. pomonella*, when foods were in close proximity to fruit, patch residency and egg laying were greater than in trees with only fruit (Averill and Prokopy 1993).

Overall results suggest that *R. indifferens* in central Washington maintains stable protein and sugar levels throughout the season because sufficient amounts of nutrients are found in cherry trees during this time. Increases in nutrient availability caused by ripening and damaged cherries later in the season apparently do not result in increased amounts of nutrients in flies. Further studies should determine the contribution of nutrients on leaf surfaces alone to fecundity of flies to test the hypothesis that nutrients on leaves are limited, because this may explain why particular feeding behaviors evolved. Studies of fly feeding and nutrient availability on native bitter cherry also would be valuable in helping understand the evolution of feeding behaviors in *R. indifferens* and how it is reflected in the fly's feeding behaviors on sweet cherry.

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